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DOI: https://doi.org/10.1111/eva.12431

Received: 3 September 2015 | Accepted: 7 September 2016

DOI: 10.1111/eva.12431

ORIGINAL ARTICLE

WILEY Evolutionary Applications Open Access

Manipulating virulence factor availability can have complex consequences for infections

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Funding information
The Swiss National Science Foundation, Grant/Award Number: PP00P3-139164; the Novartis Foundation for medical-biological research, Grant/Award Number: 14B0457; DAAD; HFSP, Grant/Award Number: RGP0011/2014

Abstract

Given the rise of bacterial resistance against antibiotics, we urgently need alternative strategies to fight infections. Some propose we should disarm rather than kill bacteria, through targeted disruption of their virulence factors. It is assumed that this approach (i) induces weak selection for resistance because it should only minimally impact bacterial fitness, and (ii) is specific, only interfering with the virulence factor in question. Given that pathogenicity emerges from complex interactions between pathogens, hosts and their environment, such assumptions may be unrealistic. To address this issue in a test case, we conducted experiments with the opportunistic human pathogen *Pseudomonas aeruginosa*, where we manipulated the availability of a virulence factor, the iron-scavenging pyoverdine, within the insect host *Galleria mellonella*. We observed that pyoverdine availability was not stringently predictive of virulence and affected bacterial fitness in nonlinear ways. We show that this complexity could partly arise because pyoverdine availability affects host responses and alters the expression of regulatorily linked virulence factors. Our results reveal that virulence factor manipulation feeds back on pathogen and host behaviour, which in turn affects virulence. Our findings highlight that realizing effective and evolutionarily robust antivirulence therapies will ultimately require deeper engagement with the intrinsic complexity of host–pathogen systems.

KEYWORDS

antivirulence therapy, *Galleria mellonella*, host effects, opportunistic pathogen, pathogen fitness, pleiotropy, *Pseudomonas aeruginosa*, siderophore

1 | INTRODUCTION

The pervasive idea that virulence—the damage a host experiences during infection—follows more or less directly from pathogen load has shaped our view of infectious disease since the early days of germ theory (Anderson & May, 1979; Bastian, 1875; Evans, 1976; Frank, 1996; Pasteur, 1880; Stearns & Koella, 2008) and has underpinned our clinical quest to eradicate harmful microbes (Allison, Brynildsen, & Collins, 2011; Dagan, Klugman, Craig, & Baquero, 2001; Russell, 2011). However, advances over the years have revealed that the severity of an infectious disease depends on much more than just the sheer number of pathogens present; rather, it derives from complex interactions between the pathogen, its host and the prevailing abiotic and biotic ecological conditions (Bull & Lauring, 2014; de Lorenzo, 2015; Méthot & Alizon, 2014; Schmid-Hempel, 2011). In other words, a microbe's pathogenicity is not so much about what it is and how abundant it is, but what it does, when it does it and to whom.

These insights have important consequences for antibacterial therapies that seek to control rather than eradicate infections (Vale et al., 2016). In particular, "antivirulence" approaches have been seen as promising alternatives to classic antibiotics (Allen, Popat, Diggle, & Brown, 2014; Cegelski, Marshall, Eldridge, & Hultgren, 2008; Rasko & Sperandio, 2010; Vale et al., 2016). Such therapies seek to disarm rather than kill pathogens and do so by inhibiting the synthesis or the functioning of virulence factors (e.g. toxins, tissue-degrading enzymes, iron-scavenging siderophores, quorum sensing signals; Rahme et al., 1995; Miethke & Marahiel, 2007; Nadal Jimenez et al., 2012; LaSarre & Federle, 2013). The appeal of this strategy is that any effects on bacterial fitness should be relatively minor, and therefore, such treatments should induce only relatively weak selection for resistance (André & Godelle, 2005; Pepper, 2012). However, given the above-mentioned complexities intrinsic in infectious diseases, we can expect that in many cases, a given antivirulence drug will have effects that extend beyond simply quenching the targeted virulence factor. We might have all sorts of unanticipated secondary effects on the behaviour of the pathogen and its host. For example, the suppression of one virulence factor could pleiotropically affect the regulation of another virulence factor due to regulatory linkage at the genetic level (Balasubramanian, Schneper, Kumari, & Mathee, 2013; García-Contreras et al., 2014; Herrera, García-Arriaza, Pariente, Escarmís, & Domingo, 2007; Nadal Jimenez et al., 2012). Furthermore, virulence factors often serve as cues for hosts to mount an immune response (Miyashita, Takahashi, Ishii, Sekimizu, & Kaito, 2015; Park et al., 2014; Schmid-Hempel, 2005; Taszlow & Wojda, 2015), so interfering with some virulence factors' availability could indirectly modulate host responses.

In the light of this inherent complexity, it seems challenging to predict how a specific antivirulence therapy will likely affect bacterial load and treatment efficacy. If indeed the treatment causes secondary effects of the sort envisaged above, we might need to carefully re-evaluate previous claims on the evolutionary robustness of such therapies. Complex interactions between pathogen and host factors could bring into play a multitude of different traits, all of which would be potential targets upon which natural selection could act on. Consequently, there could still be considerable selection for pathogen variants that are resistant to the treatment and/or become more virulent (Vale, Fenton, & Brown, 2014; Vale et al., 2016).

Here, we use the opportunistic human pathogen *Pseudomonas aeruginosa* as a test case to investigate the consequences of manipulating virulence factor availability. This bacterium relies on a number of virulence factors to establish infections in animals and humans, including immune-compromised cystic fibrosis patients (Lyczak, Cannon, & Pier, 2002; Papaioannou, Utari, & Quax, 2013; Rahme et al., 2000). One particularly well-studied virulence factor is pyoverdine, a siderophore secreted into the local environment to scavenge iron from host tissue (Cornelis & Dingemans, 2013; Harrison, Browning, Vos, & Buckling, 2006; Meyer, Neely, Stintzi, Georges, & Holder, 1996). Pyoverdine is a multifunctional molecule. It can be shared as public good between cells for iron uptake to stimulate growth and biofilm formation (Banin et al., 2008; Buckling et al., 2007). It is also used as a signalling molecule to control its own expression, and the synthesis of two additional virulence factors, exotoxin A and protease IV (Lamont, Beare, Ochsner, Vasil, & Vasil, 2002). Additionally, it can act as a toxin by interfering with mitochondrial iron homoeostasis (Kirienko, Ausubel, & Ruvkun, 2015). For all those reasons, pyoverdine has been identified as a suitable target for antivirulence therapies (Banin et al., 2008; Bonchi, Frangipani, Imperi, & Visca, 2015; Bonchi, Imperi, Minandri, Visca, & Frangipani, 2014; Kaneko, Thoendel, Olakanmi, Britigan, & Singh, 2007; Lamont et al., 2002; Ross-Gillespie, Weigert, Brown, & Kümmerli, 2014; Visca, Imperi, & Lamont, 2007). In this study, we manipulated the availability of pyoverdine in the context of experimental infections of greater waxmoth larvae (*Galleria mellonella*). We investigated how interference with this virulence factor affects (i) bacterial growth within the host; (ii) the host's response to infections; (iii) the pleiotropic regulatory links to other virulence factors; and (iv) how these factors combine and determine the overall level of virulence the host experiences. Building from previous work, we reduced the in vivo availability of pyoverdine by supplementing bacterial inocula with gallium, an iron mimic that inactivates pyoverdine molecules by binding irreversibly to them in place of iron (Kaneko et al., 2007; Ross-Gillespie et al., 2014). In addition, we also explored pathogen and host responses under conditions of increased pyoverdine availabilities. This allows us to test more generally how predictive virulence factor availability is for disease severity.

2 | MATERIALS AND METHODS

2.1 | Strains and media

Our experiments featured the clinical isolate *P. aeruginosa* PAO1 (ATCC 15692), a pyoverdine-defective knockout strain derived from this wild type (PAO1Δ*pvdD*), and three derivatives of these strains engineered via chromosomal insertion (*att*Tn7::ptac-*gfp*, *att*Tn7::ptac-*mcherry*) to constitutively express fluorescent proteins—that is PAO1-*gfp*, PAO1-*mcherry* and PAO1Δ*pvdD-gfp*. Overnight cultures were grown in 8 ml Luria–Bertani (LB) medium in 50-ml Falcon tubes and incubated at 37°C, 200 rpm for 16–18 hr. For all experiments, we subsequently diluted the overnight cultures in 0.8% NaCl saline solution. For in vitro assays, we used iron-limited CAA medium (per litre: 5 g casamino acids, 1.18 g K_2HPO_4*$3H_2O$, 0.25 g $MgSO_4$*$7H_2O$, 100 μg/ml human apotransferrin, 20 mM $NaHCO_3$ and 25 mM HEPES buffer). Human apotransferrin in combination with $NaHCO_3$ (as cofactor) is a strong iron chelator, which prevents non-siderophore-mediated iron uptake. All chemicals were purchased from Sigma-Aldrich, Switzerland. Pyoverdine was isolated using the protocol by Meyer et al. (1997).

2.2 | Manipulation of pyoverdine availability

In both our in vitro and in vivo assays, we reduced and increased pyoverdine availability by supplementing bacterial inocula with, respectively, either gallium nitrate or purified pyoverdine. Gallium is an iron mimic that inactivates pyoverdine molecules by binding irreversibly

to them in place of iron. It thereby lowers pyoverdine availability in a dose-dependent manner (Kaneko et al., 2007; Ross-Gillespie et al., 2014). The addition of pyoverdine immediately increases availability after inoculation, which has been shown to stimulate bacterial growth in vitro (Kümmerli & Brown, 2010). For in vitro experiments, we varied gallium and pyoverdine concentrations from 5 to 250 μM. For in vivo experiments, we prepared inocula with 10-fold higher concentrations, as we assumed that upon injection into a host larva's haemolymph (a total volume of approximately 100 μl; Harding, Schroeder, Collins, & Frankel, 2013), our infection inoculum (a 10 μl volume) would become diluted by a factor of approximately ten. Hereafter we report in vivo concentrations as estimated final concentrations, adjusted to reflect this assumed 10-fold dilution.

2.3 | In vitro growth and pyoverdine assays

To assess how our treatment regimes affect pyoverdine availability and bacterial growth, we performed in vitro growth assays. Overnight LB cultures (PAO1 and PAO1Δ*pvdD*) were washed twice and standardized for optical density (OD = 2) and then inoculated at 10^{-3} dilution to iron-limited CAA supplemented with either gallium nitrate ($Ga(NO_3)_3$; 5, 10, 20, 50 and 250 μM) or purified pyoverdine (same concentrations), to respectively reduce or enhance the availability of pyoverdine. All conditions were carried out in fourfold replication. Growth was tracked over 24 hr (37°C) in 200 μl cultures in 96-well plates (BD Falcon, Switzerland) using a Tecan Infinite M-200 plate reader (Tecan Group Ltd, Switzerland). We measured OD at 600 nm and pyoverdine-associated fluorescence (400 ex | 460 em), every 15 min following brief shaking of the plate (30s, 3.5 mm orbital displacement). As gallium increases pyoverdine fluorescence, we corrected fluorescence values using a previously published calibration curve (Ross-Gillespie et al., 2014).

2.4 | In vivo growth assays

Infections were performed following protocols described in Ross-Gillespie et al. (2014). Briefly, final-instar *Galleria mellonella* larvae, standardized for mass and general condition, were surface-sterilized with 70% ethanol, inoculated between the posterior prolegs (Hamilton syringe; 26s gauge sterile needle) and then individually (randomly) distributed to the wells of 24-well plate for incubation at 37°C. In vivo bacterial growth was assayed as per Ross-Gillespie et al. (2014), using GFP fluorescence signal as a proxy for growth. For this reason, we infected larvae with bacterial strains harbouring a constitutively expressed *gfp* marker (i.e. PAO1-*gfp* or PAO1Δ*pvdD-gfp*). Inocula (10 μl) contained ~25 colony-forming units (CFU) of either PAO1-*gfp* supplemented with gallium (50 μM or 250 μM) or pyoverdine (50 μM or 250 μM), no pyoverdine or the pyoverdine-defective PAO1Δ*pvdD-gfp* as a control treatment. A growth-negative control included the injection of saline solution. At 17 hr postinfection, larvae (24 per treatment) were processed to estimate their bacterial load. Approximately 50% of the larvae that had been infected with the wild-type strain were already dead at this time point. Larvae were individually flash-frozen in liquid nitrogen and then ground to fine powder using sterile micropestles. Powderized larval homogenates were resuspended in 1 ml sterile H_2O and centrifuged at 6300 RCF for 2 min. Thereafter, 200 μl of the water-soluble liquid phase of each sample was transferred to a 96-well plate and assayed for GFP-associated fluorescence using a Tecan Infinite M-200 plate reader. To examine whether the bacterial load at 17 hr postinfection is representative of within-host growth dynamics, we repeated the experiment for a subset of treatments (untreated wild type, wild type with intermediate (50 μM) gallium or pyoverdine concentration, pyoverdine-deficient mutant, saline control). At 13, 15, 17 and 20 hr, we processed randomly selected larvae (24 per treatment) as described above and measured their bacterial load.

2.5 | Ex vivo growth assays

We investigated the potential influence of host effects on bacterial dynamics via ex vivo growth assays in haemolymph. In a first step, we primed *G. mellonella* larvae by inoculating them with bacterial wild-type cultures featuring manipulated levels of pyoverdine (by supplementing inoculum with either intermediate (50 μM) concentrations of gallium or pyoverdine). As controls, we primed larvae by infecting them with either the pyoverdine-deficient strain, pyoverdine alone, heat-killed wild-type bacteria or the saline control. In a second step, we then measured bacterial growth in haemolymph extracted from these primed larvae. The priming inocula were administered as per the infection protocol described above. Inoculated larvae were distributed, in groups of 4, to petri dishes and incubated at 37°C. After 14 hr, the petri dishes were placed on ice for 15 min to anaesthetize the larvae prior to haemolymph extraction. A small incision was made in the posterior segment using a sterile scalpel, and haemolymph was drained with the aid of gentle pressure (Harding et al., 2013). From each sample, 25 μl of haemolymph was immediately stabilized with 15 μl of an ice-cold pH 6.5 cacodylate buffer (10 mM $Na\text{-}C_2H_7AsO_2$ and 5 mM $CaCl_2$) and 15 μl of a saturated propylthiouracil solution to inhibit melanization. Samples were then centrifuged (514 RCF, 2 min) to separate the liquid haemolymph fraction from any solid tissue contaminants, and 30-μl aliquots were transferred to individual wells of a 96-well plate, each containing 70 μl of saline solution. To kill the priming strains and any other bacteria that may have been present in the haemolymph as part of the natural larval microbiota, we added gentamicin to the haemolymph/buffer mixture to a final concentration of 20 μg/ml (a concentration known to kill susceptible *P. aeruginosa*; Choi et al., 2005). Subsequently, we inoculated wells with bacteria from an overnight culture (adjusted to an OD = 2 and subsequently diluted to 10^{-4}) of a gentamicin-resistant PAO1-*mcherry* strain (this strain showed the same growth pattern as the untagged wild-type strain). The plate was transferred to a Tecan Infinite M-200 plate reader for 24 hr of incubation at 37°C. Every 15 min, we measured cell density (measured via the *mCherry*-associated fluorescence: 582 ex | 620 em; note: using optical density as a proxy for cell density is not reliable in this naturally turbid medium). These experiments allowed us to ascertain (i) whether bacterial growth in haemolymph is affected by a host's history of prior infection and (ii) whether the availability of pyoverdine

during priming predicts subsequent bacterial growth. Note that residual pyoverdine from the priming inocula was below detection limit after haemolymph extraction, and therefore should not influence later bacterial growth patterns.

2.6 | Molecular investigation of pyoverdine-mediated pleiotropy

Because pyoverdine is not only a virulence factor but also a signalling molecule, manipulating pyoverdine availability might also affect, via interaction with the PvdS iron-starvation sigma factor, the production of two additional virulence factors, exotoxin A and protease IV (Lamont et al., 2002; Ochsner, Johnson, Lamont, Cunliffe, & Vasil, 1996; Wilderman et al., 2001). We used qPCR to explore whether our extrinsic manipulation of pyoverdine levels could change pyoverdine-mediated signalling and therefore pleiotropically affect expression of genes for virulence factor production (*pvdS*, *toxA* coding for exotoxin A, *prpL* (alternative name: *piv*) coding for protease IV and *pvdA* coding for one of the pyoverdine synthesis enzymes). PAO1 cells were grown until early- and mid-exponential growth phases in 20 ml standard CAA (in a sterile 500-ml Erlenmeyer) containing either (i) no supplement, (ii) 10 μM $Ga(NO_3)_3$ (to reduce pyoverdine availability), (iii) 200 μM purified pyoverdine (to increase pyoverdine availability) or (iv) 100 μM $FeSO_4$ (our negative control under which pyoverdine production should be completely switched off; Kümmerli, Jiricny, Clarke, West, & Griffin, 2009). RNA was extracted using a modified hot acid phenol protocol and purified as in Pessi et al. (2007, 2013). Residual DNA in the sample was eliminated using RQ1 RNase-free DNase I, and purification was performed using the RNeasy Mini Kit (Qiagen). Absence of DNA was verified by PCR using the primers specified in Table S1, and 40 cycles with the GoTaq Polymerase (Promega, Switzerland). RNA quality in the purified samples was then assessed using RNA Nano Chips (Agilent 2100 Bioanalyzer; RIN (RNA integrity number) >7.6). First-strand cDNA synthesis with 10 μg of total RNA from each sample was performed with M-MLV reverse transcriptase RNase H Minus (Promega) and random primers (Promega). cDNA was subsequently purified with the MinElute PCR Purification Kit (Qiagen). The expression of *Pseudomonas aeruginosa* PAO1 genes PA2399 (*pvdD*), PA2426 (*pvdS*), PA1148 (*toxA*) and PA4175 (*prpL)* was analysed with a Stratagene MX300P instrument (Agilent) using GoTaq qPCR Master Mix (Promega). All PCRs were analysed in triplicate with three cDNA dilutions (15, 7.5 and 3.25 ng) and 0.4 μM of individual primers in a total volume of 25 μl per reaction. Primers (Table S1) were designed using Primer3Plus (Untergasser et al., 2007) and subsequently verified by PCR. Fold changes were calculated using the ΔΔCT method (Pfaffl, 2001) using the primary sigma factor *rpoD* (PA0576) as housekeeping gene for data normalization.

2.7 | Virulence assays

Infections were performed as described above. Inocula (10 μl) contained ~25 CFU of *P. aeruginosa* from an overnight culture, resuspended in saline solution and supplemented with either gallium nitrate (5 μM, 50 μM, 250 μM), pyoverdine (10 μM, 50 μM, 250 μM) or neither. Controls included saline-only, gallium-only (50 μM and 250 μM) and pyoverdine-only inocula (50 μM and 250 μM), and also the PAO1Δ*pvdD* strain, defective for pyoverdine production. The vitality of all larvae (i.e. spontaneous movement/response to tactile stimulation) was assessed hourly, starting at 10 hr postinjection. Some of the larvae (n = 25, 3.14%) either started pupating while under observation or died prematurely during the first 10 hr postinjection—presumably as a result of handling—and hence were excluded from further analyses.

2.8 | Statistical analysis

We used the functions from the "grofit" R package to fit spline curves to the growth and pyoverdine production trajectories. From these fitted curves, we extracted growth parameters. In particular, we focused on growth integrals (areas under curves), which combine information from the lag phase, growth rate and yield. Growth integrals are particularly useful for nonlogistic growth trajectories as observed throughout our experiments.

Survival curves were analysed by fitting parametric Weibull survival curves with the aid of functions from the "survival" R package (Therneau & Grambsch, 2000). From the fitted models, we extracted the hazard ratios and used those values to estimate the mortality risk of larvae within each treatment. To confirm the robustness of our analysis, we also performed Cox proportional hazards regression, which yielded qualitatively similar results.

We used both parametric and nonparametric statistical models to test for treatment effects. Specifically, we used Kendall rank correlation analyses to test for associations between pyoverdine availability, growth, host response and virulence. The data from our in vivo and ex vivo growth experiments did not meet the criteria of normally distributed residuals and the homogeneity of variances, which precluded the use of parametric statistical tests. For these analyses, we used the nonparametric Kruskal–Wallis test. All analyses were performed in R 3.3.0 (R Development Core Team 2015).

3 | RESULTS

3.1 | Treatment effects on in vitro bacterial pyoverdine availability and growth

We first tested whether our treatment regime (i.e. adding gallium to quench pyoverdine or supplementing additional pyoverdine) indeed altered pyoverdine availability as intended. We found that our treatment regime had a positive linear effect on pyoverdine availability (Figure 1a; Kendall's correlation coefficient: $\tau = .75$, $p < .001$, measured during the first 8 hr of the growth period when pyoverdine is most needed to overcome iron limitation; Kümmerli & Brown, 2010). Moreover, we found that our manipulation of pyoverdine availability had a significant linear effect on bacterial growth (Figure 1b; $\tau = .93$, $p < .001$): adding gallium reduced growth, while pyoverdine

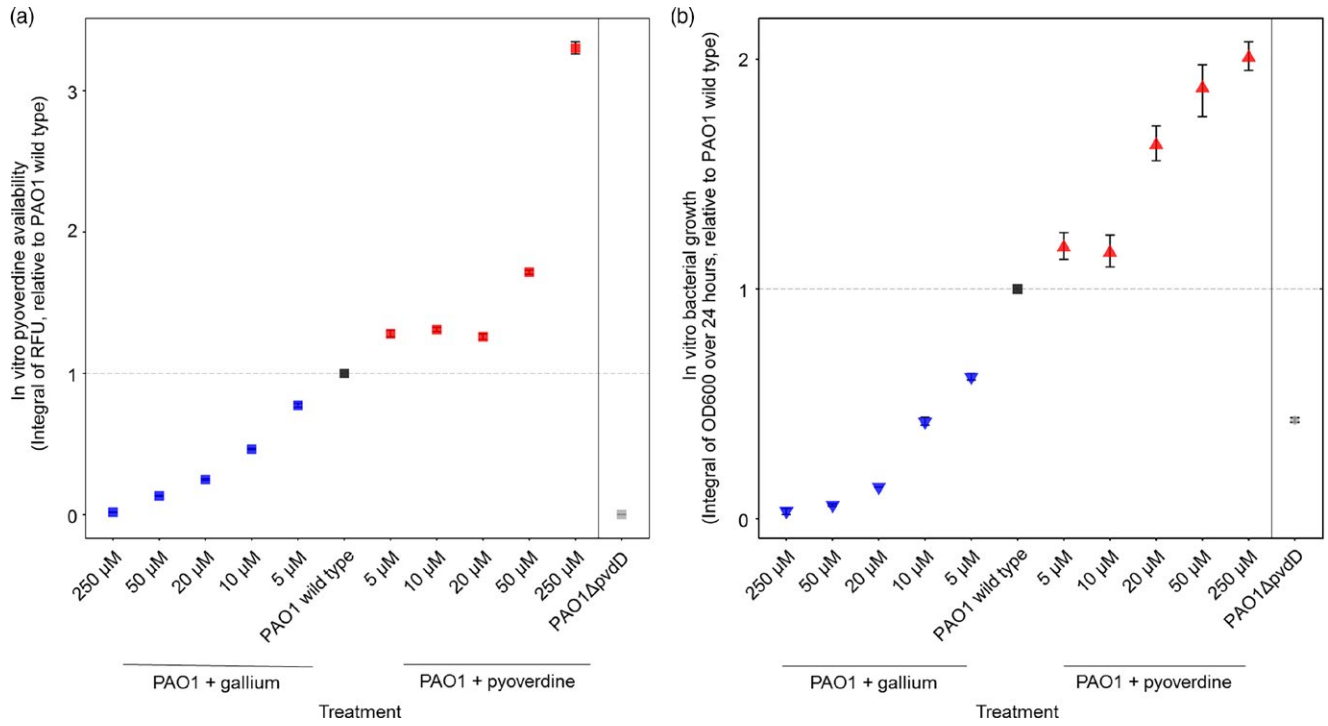

FIGURE 1 Our treatment regime significantly affected pyoverdine availability (a) and bacterial growth (b) in linear ways. To capture the dynamics of pyoverdine availability (first 8 hr) and growth (24 hr) in bacterial cultures, we used integrals (i.e. area under the curve) for analysis. Nonlinear patterns in (a), for example for the 10 µM and 20 µM pyoverdine supplementations, can arise because bacteria plastically adjust their pyoverdine production level according to their need (Kümmerli et al., 2009), such that de novo production and supplementation can balance each other out over time. Symbols and error bars represent mean estimates and 95% confidence intervals, respectively, across four independent replicates

supplementation accelerated growth relative to the unsupplemented wild type. Taken together, our in vitro experiments show that our treatment scheme successfully manipulates pyoverdine availability and that pyoverdine is a growth promoter, essential for bacteria to thrive in iron-limited medium.

3.2 | Nonlinear effects of pyoverdine availability on in vivo bacterial growth

Pyoverdine availability also had significant effects on bacterial growth within the *G. mellonella* larvae (Kruskal–Wallis test for differences between treatments: $\chi^2 = 34.80$, $p < .001$; Figure 2), but the overall effect was not linear. Instead, bacterial load peaked in infections with the unsupplemented wild type (i.e. at intermediate pyoverdine availability). Both the addition of gallium and pyoverdine significantly reduced bacterial growth compared to unsupplemented wild-type infections (for gallium 50 and 250 µM combined: $\chi^2 = 8.68$, $p = .013$; for pyoverdine 50 and 250 µM combined: $\chi^2 = 6.66$, $p < .010$). Bacterial growth also significantly peaked in infections with the unsupplemented wild type when considering the entire growth trajectories and not only a single time point (Fig. S1), thereby confirming the above pattern.

One possible explanation for the absence of a linear relationship between pyoverdine availability and in vivo growth is that pyoverdine might not be required for bacteria to thrive within the host. However, two control experiments speak against this hypothesis. First, the growth of a pyoverdine-deficient knockout strains was significantly impaired in host infections compared to the wild type (Kruskal–Wallis test: $\chi^2 = 7.54$, $p < .001$; Figure 2). Second, ex vivo growth of wild-type bacteria in extracted haemolymph demonstrated significant iron limitation and high pyoverdine production in this medium (Fig. S2). Altogether, these results indicate that pyoverdine is important for iron scavenging and growth within the larvae.

3.3 | Pyoverdine availability affects host responses

To investigate whether bacteria and/or pyoverdine availability triggers variation in host responses, we tracked growth of a wild-type strain ex vivo in haemolymph extracts from larvae previously primed under different conditions. Ex vivo bacterial growth in haemolymph indeed significantly differed depending on the infection history of the larvae (Figure 3; Kruskal–Wallis test: $\chi^2 = 10.59$, $p = .014$, including the pyoverdine manipulation regimes and the saline control). Specifically, bacteria showed significantly lower growth in haemolymph from wild-type-primed larvae than in haemolymph from saline-primed larvae ($\chi^2 = 4.11$, $p = .043$). Furthermore, we found a significant negative association between the availability of pyoverdine in the priming inocula and the subsequent ex vivo bacterial growth (Figure 3; Kendall's $\tau = -.21$, $p = .023$). Control experiments revealed that a significant host response can be triggered by multiple stimuli: priming larvae

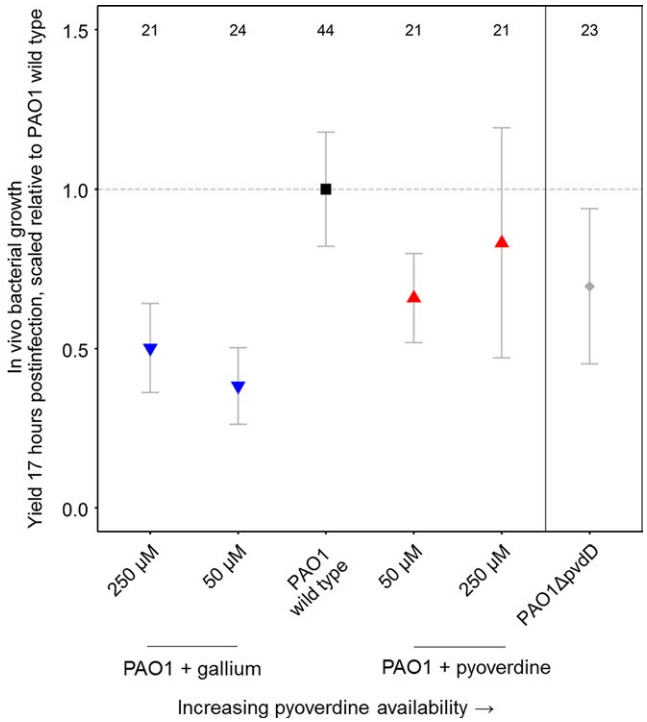

FIGURE 2 Pyoverdine availability has nonlinear effects on *P. aeruginosa* growth in *G. mellonella* larvae. Bacterial load (measured 17 hr postinfection) peaked in infections with the unsupplemented wild type, while the supplementation of both gallium (blue) and pyoverdine (red) resulted in a significant drop of bacterial load. Infections with a pyoverdine-deficient strain also resulted in a significant growth reduction compared to wild-type infections, indicating that pyoverdine is important for growth in this host. Symbols and error bars represent mean estimates and 95% confidence intervals, respectively. Numbers on top show sample size for each treatment

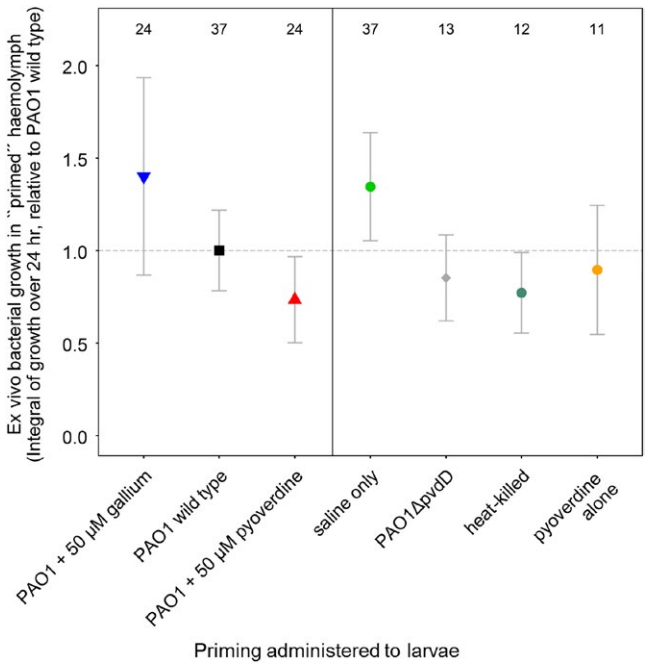

FIGURE 3 Growth of *P. aeruginosa* in haemolymph extracted from primed larvae demonstrates context-dependent host effects. Fourteen hours prior to haemolymph extraction, larvae were primed with wild-type bacteria, either alone or supplemented with gallium or pyoverdine. Control larvae were primed with saline alone, pyoverdine alone, heat-killed wild-type bacteria or a pyoverdine-deficient strain. Haemolymph extracts were gentamicin-treated to kill previously inoculated bacteria, and growth assays were then performed with a gentamicin-resistant wild-type strain. Compared to the saline control, haemolymph primed with wild-type bacteria was significantly more refractory to subsequent bacterial growth, demonstrating a host response to infection. Moreover, we found a significant negative correlation between pyoverdine availability during the priming phase and the subsequent bacterial growth, indicating that pyoverdine is involved in triggering host responses. Symbols and error bars represent mean estimates and 95% confidence intervals, respectively. Numbers on top show sample size for each treatment

with non-pyoverdine-producing bacteria, pyoverdine alone or heat-killed bacteria all resulted in a similarly increased response relative to the saline priming (Kruskal–Wallis test comparing pooled control treatments versus the saline treatment: $\chi^2 = 8.24$, $p = .004$). Overall, our findings suggest that haemolymph primed with bacteria has a growth-inhibiting effect on *P. aeruginosa* and that this effect can vary plastically over time in response to pyoverdine availability.

3.4 | Pyoverdine availability affects the expression of other virulence factors

In addition to its function as a siderophore, pyoverdine is also a signalling molecule, which controls its own production and the synthesis of two other virulence factors, namely protease IV and exotoxin A (Beare, For, Martin, & Lamont, 2002; Lamont et al., 2002) (Figure 4). It is therefore well conceivable that the experimental manipulation of pyoverdine availability also affects the expression of these other virulence factors. To test this hypothesis, we performed in vitro qPCR experiments, following the expression of the genes *pvdS*, *pvdA*, *prpL* and *toxA* across three levels of pyoverdine availabilities and two time points (early- and mid-exponential phase). We examined these time points because pleiotropy relatively early in the growth cycle is likely to have the biggest effect on subsequent pathogen growth and virulence. The four genes code for the sigma factor PvdS (the main regulator of all three virulence factors), PvdA (enzyme involved in pyoverdine synthesis), protease IV and exotoxin A (Figure 4). Taking the unsupplemented wild-type bacteria growing in our standard iron-limited medium as a reference, we found that the addition of iron dramatically downregulated the expression of all four genes (Table 1). This suggests that all three virulence factors (pyoverdine, protease IV and exotoxin A) are significantly expressed under the imposed iron-limited conditions (see also Ochsner, Wilderman, Vasil, & Vasil, 2002). Next, we examined whether gene expression levels change as a function of pyoverdine availability. We found that pyoverdine manipulation either did not affect gene expression or resulted in the downregulation of interlinked genes (Table 1). As there were no marked differences in gene expression profiles between the early- and the mid-exponential growth phase,

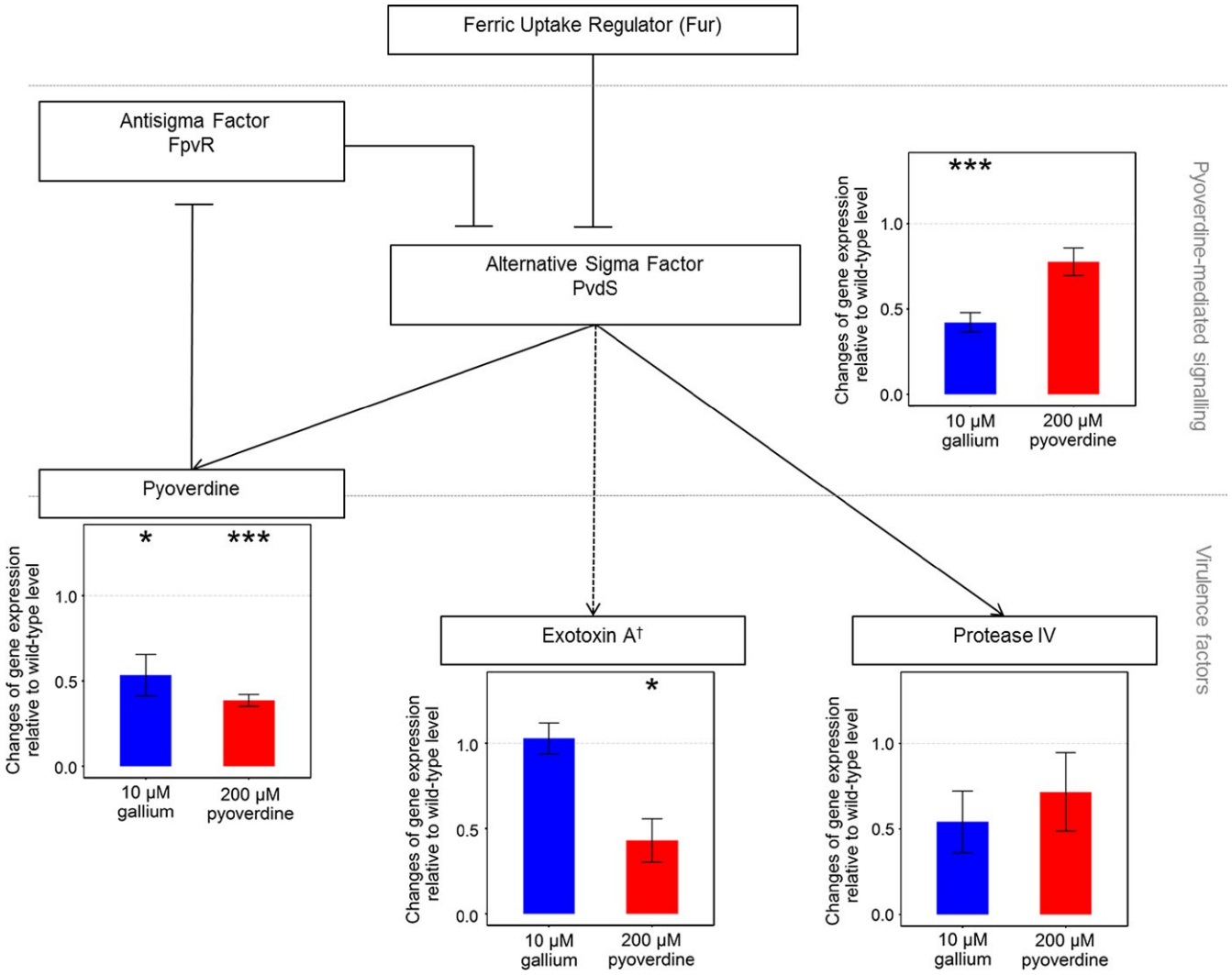

FIGURE 4 Manipulating pyoverdine availability has moderate effects on the pyoverdine signalling network, and generally leads to the downregulation of interlinked genes. Pyoverdine production is controlled by the alternative sigma factor PvdS that is itself negatively regulated by the ferric uptake regulator FUR, in response to intracellular iron levels. Pyoverdine modulates PvdS activity through a signalling cascade. Incoming iron-bound pyoverdine binds to its cognate receptor, thereby triggering the lysis of the membrane-bound antisigma factor FpvR, which binds and inhibits PvdS. In turn, membrane-released PvdS triggers increased transcription of pyoverdine synthesis genes, but also activates the expression of prpL (encoding the protease IV virulence factor) and toxR (coding for the ToxR regulator that then stimulates the expression of exotoxin A). Reduced pyoverdine availability (gallium supplementation, blue bars) moderately but significantly reduced pvdS and pvdA expression. Increasing pyoverdine availability (red bars) moderately but significantly reduced pvdA and toxA expression. Shown are mean values and standard errors across four replicates. Asterisks indicate significant gene expression changes relative to the unsupplemented wild type ($p < .05$). † indirect regulation via toxR regulator

we pooled the data to identify the genes that were significantly downregulated (Figure 4). These analyses revealed that the addition of gallium (10 µM) slightly but significantly reduced the expression of *pvdS* ($t_3 = -10.55$, $p = .002$) and *pvdA* ($t_3 = -3.87$, $p = .031$). The supplementation of pyoverdine (200 µM) significantly reduced the expression of *pvdA* ($t_3 = -17.95$, $p < .001$) and *toxA* ($t_3 = -4.50$, $p = .020$). Our results are promising from a therapeutic perspective, as they suggest that the manipulation of pyoverdine availability does not increase the expression of the interlinked virulence factors protease IV and exotoxin A, but rather has a neutral or even a negative effect on their expression.

3.5 | Relationship between pyoverdine availability and virulence

Our results presented above (Figures 2–4) show that the manipulation of pyoverdine availability has nonlinear effects on bacterial load, triggers differential host responses and has slight pleiotropic effects on the expression of other virulence factors. How do these factors now all combine within the host and determine the overall level of virulence associated with pyoverdine manipulation? Overall, our experimental infections of *G. mellonella* larvae revealed a significant positive association between pyoverdine availability and virulence

TABLE 1 Expression fold changes for *P. aeruginosa* genes involved in pyoverdine-mediated signalling

		Supplementation regime					
		100 μM FeSO4		10 μM $Ga(NO_3)_3$		200 μM Pyoverdine	
Growth Phase	Gene	Mean	SE	Mean	SE	Mean	SE
Early-exponential	*pvdS*	0.0003	0.0002	0.3256	0.0199	0.7237	0.1719
	pvdA	0.0001	0.0000	0.3442	0.0353	0.3675	0.0159
	toxA	0.0544	0.0098	0.9063	0.0161	0.2154	0.0200
	prpL	0.2321	0.0375	0.7072	0.3661	1.1106	0.0821
Mid-exponential	*pvdS*	0.0003	0.0002	0.5167	0.0350	0.8273	0.0628
	pvdA	0.0003	0.0002	0.7242	0.1163	0.4059	0.0775
	toxA	0.0561	0.0092	1.1514	0.1412	0.6463	0.0519
	prpL	0.0214	0.0046	0.3776	0.0896	0.3240	0.0143

Expression fold changes of *pvdS* (encoding the iron-starvation sigma factor PvdS), *pvdA* (coding for one of the pyoverdine synthesis enzymes), *toxA* (coding for exotoxin A) and *prpL* (encoding protease IV) are expressed relative to the unsupplemented PAO1 wild-type regime.

(Figure 5; Kendall's τ = .71, p = .030). Larvae died earlier in infections supplemented with pyoverdine, but survived longer when gallium was added instead. However, the trend was not altogether monotonic: moderately increased pyoverdine availability (10 μM) significantly decreased rather than increased the virulence risk (parametric survival regression assuming Weibull distribution: coefficient = 0.082 ± 0.028, mean ± SE, z = 2.89, p = .004). Such low-pyoverdine-supplementation infections showed virulence levels comparable to those of unsupplemented infections involving the pyoverdine-deficient PAO1Δ*pvdD* mutant (coefficient = 0.0055 ± 0.028, z = 1.94, p = .846). The gallium-supplemented treatments were not ordered monotonically with respect to virulence, in that high (250 μM)-gallium-supplemented infections were no less virulent than intermediate (50 μM)-gallium-supplemented infections (coefficient = 0.043 ± 0.037, z = 1.18, p = .238).

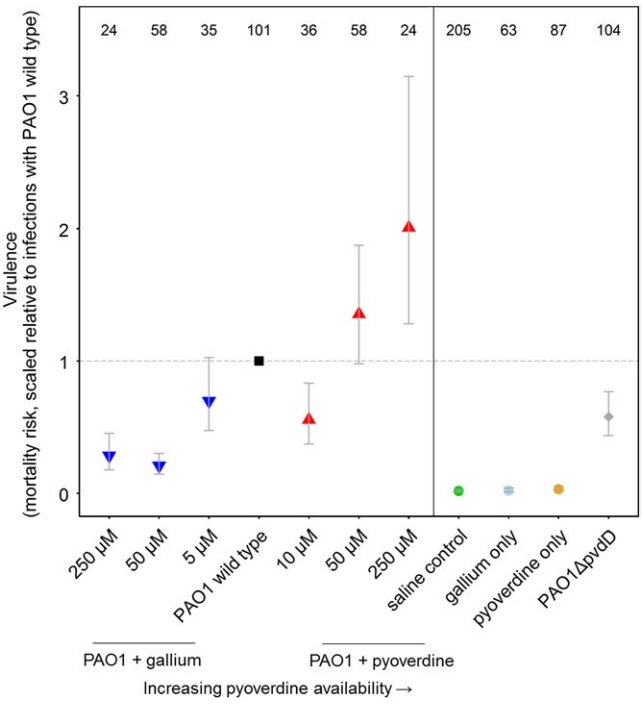

FIGURE 5 Relationship between pyoverdine availability and virulence, measured as mortality risk of larvae within each treatment. Overall, there is a positive correlation between pyoverdine availability and virulence with a notable exception. Supplementing the infection with 10 μM pyoverdine reduced virulence comparable to a pyoverdine-deficient mutant. Symbols and error bars represent mean estimates and 95% confidence intervals, respectively. Numbers on top show sample size for each treatment

4 | DISCUSSION

Our results show that the manipulation of pyoverdine, an important virulence factor of the opportunistic human pathogen *P. aeruginosa*, affects bacterial load in infections of *G. mellonella* larvae in complex ways, triggers differential host responses and influences the expression of other regulatorily linked virulence factors (Figures 2–4). Our findings have important consequences for recently proposed antivirulence therapies, targeting pyoverdine-mediated iron uptake (Bonchi et al., 2014, 2015; Imperi et al., 2013; Kaneko et al., 2007; Ross-Gillespie et al., 2014), because complex interactions between bacterial load, host response and regulatory pleiotropy could result in unpredictable treatment outcomes (García-Contreras et al., 2014). We examined this possibility for our system and found an overall positive relationship between pyoverdine availability and virulence, but also notable deviations from a monotonic pattern. For instance, the supplementation of low levels of pyoverdine significantly decreased rather than increased virulence, with this treatment reaching virulence levels comparable to infections with the pyoverdine knockout strain (Figure 5).

Some of the discovered complex nonlinear associations between bacterial load, host response, pleiotropy and virulence warrant closer examination. For instance, why does increased pyoverdine availability (50 μM and 250 μM supplementation regimes) increase virulence despite the fact that these treatments reduce bacterial growth in

vivo (Figure 2) and results, in vitro at least, in the downregulation of the coupled virulence factor exotoxin A (Figure 4)? One possible explanation is that high pyoverdine supplementation triggers an excessive host response, which is not only curbing bacterial growth, but is also damaging the host itself. For instance, *G. mellonella* produces the iron chelator transferrin as part of its innate immune response (Han, Nam, Seo, & Yun, 2004), a protein which actively counteracts the iron-scavenging activities of pathogens (Miethke & Marahiel, 2007). Such a host response typically entails costs in terms of metabolic burden and autoimmune damage, and therefore must be appropriately calibrated (Day, Graham, & Read, 2007; Medzhitov, Schneider, & Soares, 2012). An overreaction from the host, perhaps in response to a high concentration of pyoverdine, could actually exacerbate, rather than reduce, virulence. Important to note is that although pyoverdine seems to induce a host response (Figure 3), it is not toxic itself, as larvae infected with pyoverdine alone all remained healthy (Figure 5).

Another complex association was that when increasing pyoverdine availability a little bit (10 μM) compared to the wild-type treatment, we observed a significant reduction of virulence (Figure 5). This drop can potentially be explained by a host response too, but this time by a well-calibrated one, which primarily harms the pathogen while being beneficial for the host. If this explanation holds true, then the supplementation of moderate amounts of pyoverdine could represent a treatment that boosts host tolerance. Interestingly, treatments that increase host tolerance have, in addition to antivirulence approaches, been proposed as alternative ways to combat infections (Ayres & Schneider, 2012; Medzhitov et al., 2012; Vale et al., 2014, 2016).

Finally, we observed that infections with intermediate amounts of gallium (50 μM) were significantly less virulent than infections with the pyoverdine-deficient knockout strain (Figure 5). This suggests that this treatment has other effects, in addition to simply depriving siderophores from pathogens. One explanation would be that gallium has some general toxicity towards bacteria beyond its role in inhibiting iron uptake (Bonchi et al., 2014). An alternative explanation, which is supported by our previous findings (Ross-Gillespie et al., 2014) but also the qPCR data (Figure 4), is that intermediate gallium levels maintain pyoverdine synthesis, while high gallium levels completely stall the production. This steady production likely imposes a twofold cost on bacteria: gallium does not only prevent pyoverdine-mediated iron uptake, but also induces continuous replacement of pyoverdine, which likely demands a high metabolic investment for very little reward (because pyoverdine is quenched by gallium once secreted). Given the ubiquity of linkages and feedback loops in the genetic architecture of bacteria (Dumas, Ross-Gillespie, & Kümmerli, 2013; Fazli et al., 2014; García-Contreras et al., 2014; Nadal Jimenez et al., 2012), such features are likely important contributors to nonadditive effects between pathogen behaviour, fitness and virulence.

Given the complexities of host–pathogen relationships we have highlighted in this study, what could be the evolutionary consequences for antivirulence therapies? The central tenet of this approach was that disarming rather than killing pathogens should induce weaker selection for resistance because it exerts only minimal effects on pathogen fitness (André & Godelle, 2005; Pepper, 2008; Rasko & Sperandio, 2010; Stanton, 2013). Our study demonstrates that antivirulence approaches can in fact substantially modulate pathogen fitness (Figure 2; see also Liu et al., 2008), which clearly offers natural selection the opportunity to favour pathogen variants that are partially or fully resistant to the treatment (see Maeda et al., 2012; Ross-Gillespie et al., 2014; Allen et al., 2014; for detailed discussion). One obvious evolutionary response of pathogens in response to virulence factor quenching is to overproduce the virulence factor in question in order to outpace the quenching activity of the drug. Our results indicate that such an adaptation could affect the host in two different ways. If the increase in virulence factor production is substantial, this could lead to the evolution of a more virulent pathogen, which causes increased damage to the host in the absence of the treatment. Conversely, if the increase in virulence factor production is relatively small, then it could positively stimulate host responses, which in turn could curb virulence. Evolutionary responses leading to increased virulence factor production would likely involve the modification of regulatory elements. As evidenced by our study, regulatory elements can not only affect the expression of the targeted virulence factor, but also modify the expression of additional linked virulence factors in the same regulatory network (see Figure 4). How exactly such regulatory linkage would alter global virulence factor expression profiles of a pathogen in a host and how this feeds back on virulence cannot easily be foreseen, and might vary in response to the specific host stimuli present in an infection (Park et al., 2014). Finally, there might also be variation between host individuals regarding the extent to which they can cope with altered virulence factor expression. Conceivably, for an immunocompromised host even a minor overexpression of virulence factors might be fatal, whereas a healthy host might be more tolerant and easily able to cope with higher virulence factor levels. Taken together, our considerations show that we still have very limited understanding of the evolutionary consequences of antivirulence therapies. There is definitely a great need for controlled experimental evolution studies that measure selection pressures, adaptation patterns and host responses at both the proximate and ultimate level.

Given our dwindling supply of new antimicrobials, and the increasing prevalence of resistance to those we already have (Fischbach & Walsh, 2009; Levy & Marshall, 2004), creative approaches such as antivirulence therapies are certainly required (Perron, Inglis, Pennings, & Cobey, 2015; Ross-Gillespie & Kümmerli, 2014). To turn these ideas into effective and robust clinical therapies, however, we must delve deeper into the complexity of host–pathogen systems.

ACKNOWLEDGEMENTS

We thank Martina Lardi for technical assistance and three anonymous reviewers for constructive comments. RK was supported by the Swiss National Science Foundation (grant no. PP00P3-139164) and the Novartis Foundation for medical-biological research (14B0457); MW was supported by DAAD; and SPB was supported by HFSP (grant RGP0011/2014).

DATA ARCHIVING STATEMENT

Data available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.8ks41

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.